Molecular Dissection of Purinergic P2X Receptor Channels

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ABSTRACT: The P2X receptors (P2XRs) are a family of ATP-gated channels expressed in the plasma membrane of numerous excitable and nonexcitable cells and play important roles in control of cellular functions, such as neurotransmission, hormone secretion, transcriptional regulation, and protein synthesis. P2XRs are homomeric or heteromeric proteins, formed by assembly of at least three of seven subunits named $P2X_1$-$P2X_7$. All subunits possess intracellular N- and C-termini, two transmembrane domains, and a relatively large extracellular ligand-binding loop. ATP binds to still an unidentified extracellular domain, leading to a sequence of conformational transitions between closed, open, and desensitized states. Removal of extracellular ATP leads to deactivation and resensitization of receptors. Activated P2XRs generate inward currents caused by Na^+ and Ca^{2+} influx through the pore of channels, and thus mediate membrane depolarization and facilitation of voltage-gated calcium entry in excitable cells. No crystal structures are available for P2XRs and these receptors have no obvious similarity to other ion channels or ATP binding proteins, which limits the progress in understanding the relationship between molecular structure and conformational transitions of receptor in the presence of agonist and after its washout. We summarize here the alternative approaches in studies on molecular properties of P2XRs, including heteromerization, chimerization, mutagenesis, and biochemical studies.

KEYWORDS: ATP; purinergic receptors; P2X; agonist binding domain; gating; deactivation; desensitization; resensitization; chimeras

INTRODUCTION

ATP and UTP can be released by cells to act as extracellular messengers and are hydrolyzed by ecto-nucleotidases, resulting in the formation of the respective nucleoside and free phosphate. The products of this hydrolytic cascade—ADP, UDP, and adenosine—can also act as extracellular messengers by activating distinct plasma membrane receptors in numerous excitable and nonexcitable cells.[1] These receptors

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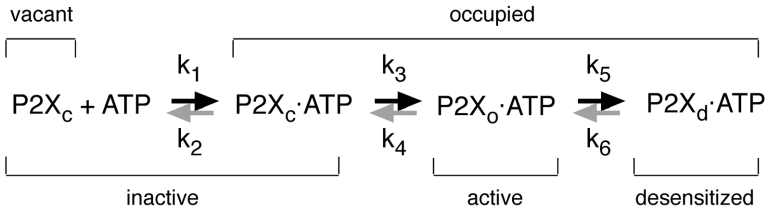
were termed purinergic and they belong to two groups: P1 receptors (P1Rs), which are activated by adenosine, and P2 receptors (P2Rs), which recognize mainly ADP, ATP, UDP, and UTP. Two families compose P2Rs: the G protein–coupled receptor family (P2YRs) and the ligand-gated channel family (P2XRs).[2] Molecular cloning revealed the expression of seven P2XR subtypes in mammals, denoted $P2X_1R$ to $P2X_7R$, and several spliced forms of these receptors. All P2XR subunits are composed of two transmembrane domains, placing most of the protein extracellularly and the N- and C-termini intracellularly. From the N-termini through the second transmembrane domain, the cloned subunits exhibit relatively high level of amino acid sequence homology. In contrast, the C-termini vary in length and show no apparent sequence homology, except for the region nearest to the second transmembrane domain.[3]

The trimeric assembly of P2XR subunits most likely accounts for formation of functional channels.[4] P2XR can form ion-permeable pores through homo- and heteromerization. $P2X_1R$, $P2X_2R$, and $P2X_3R$ subunits co-assemble in all combinations; $P2X_5R$ and $P2X_6R$ frequently co-assemble with $P2X_1R$, $P2X_2R$, and $P2X_3R$; whereas $P2X_4R$ and $P2X_7R$ form only homomeric channels.[5,6] Heteromeric receptors develop new functions resulting from integrative effects of the participating extracellular and C-terminal subdomains.[7,8] It has been suggested that two to three molecules of ATP are required to activate receptor.[9] This suggestion is consistent with a hypothesis that each subunit has one ATP binding domain.[10] Binding of ATP leads to a sequence of conformational transitions between closed, open, and desensitized states. In general accordance with del Castillo and Katz model,[11] the conformation changes for P2XRs upon ATP binding could be simplified as shown in scheme 1:



SCHEME 1.

where ($k_1/k_2 = K_A$) defines *binding*; ($k_3/k_4 = K_E$) defines *gating*, ($k_5/k_6 = K_D$) defines *desensitization* of P2XRs, and ($K_A/1+K_E$) defines the EC_{50} value for ATP. In this scenario, a transition from $P2X_o$·ATP to $P2X_c$ conformation state is termed *deactivation*, and a transition from $P2X_d$·ATP to $P2X_c$ conformation state is termed *recovery from desensitization* or *resensitization.*[12]

The current profile evoked by prolonged agonist application is commonly used as a measure of desensitization, whereas the kinetics of current decay evoked by washout of agonists is used as the measure of receptor deactivation. Both profiles were usually fitted by a single exponential function ($y = A \exp(-t/\tau) + P$) or by the sum of two exponentials ($y = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) + P$), where A_1 and A_2 are relative amplitudes of the first and second exponential, τ_1 and τ_2 are time constants, and P is plateau. The derived time constants for desensitization were labeled as τ_{des} and

for current decay after agonist withdrawal as τ_{off}. The recovery from desensitization was usually fitted using equation $I = I_{max}\ [1 - \exp(t/\tau_{rec})]$, where I is the observed peak current response, I_{max} is the maximum peak current recovery, t is the washing time and τ_{rec} is the recovery time constant.

MOLECULAR DETERMINANTS OF AGONIST BINDING DOMAIN

All P2XRs are activated by ATP, indicating the presence of a common ligand-binding domain. However, receptors differ in their sensitivity for ATP with the EC_{50}s in order: $P2X_1R = P2X_3R < P2X_2R < P2X_4R = P2X_5R < P2X_6R << P2X_7R$, suggesting the specificity of ATP binding pocket among homomeric receptors. There are other pharmacological distinctions among receptors, such as the sensitivity to αβ-methylene-ATP (αβ-meATP) and antagonists,[13] further supporting the view about the structural specificity of the ligand-binding pocket. Thus, $P2X_1R$ and $P2X_3R$ exhibit high sensitivity for αβ-meATP, which is preserved in heteromeric configurations. The sensitivity of $P2X_3R$ to αβ-meATP can also be transferred to $P2X_2R$ subtypes by generating chimeras having the ectodomain of the $P2X_3$ subunit in the $P2X_2$-based backbone.[14] It appears that the N-terminal half of $P2X_3$ ectodomain, from V60 to R180, is required to preserve high αβ-meATP sensitivity of the receptor.[7] The $P2X_2$-based chimera containing the first transmembrane domain of $P2X_1R$ or $P2X_3R$ also preserves αβ-meATP sensitivity of the receptor.[15]

Since motifs present in other nucleotide-binding proteins are absent in P2XRs and there is little sequence similarity between P2XRs and other ATP-sensitive binding proteins, the main work on identification of ATP binding domain of P2XR has been focused on conserved residues in ectodomain using the site-directed mutagenesis. The ongoing search for potentially relevant residues was based on a common finding with other ATP binding proteins, where negatively charged residues coordinate ATP-Mg^{2-} complex, positively charged residues bind the phosphate chain of ATP, and aromatic residues bind the adenine ring of ATP. Evans' group used human $P2X_1R$ as a receptor model and identified several negatively and positively charged residues,[16,17] as well as aromatic amino acids[18] that could coordinate the binding of ATP. North's group identified eight from 30 conserved charged or polar residues in the ectodomain and the first membrane-spanning region of $P2X_2$ subunit, whose mutations affected the affinity of the receptor for ATP, and seven residues, whose replacement resulted in non-functional channels.[19,20] Nakazawa and colleagues identified several additional rat $P2X_2$ ectodomain residues of the potential relevance for ATP binding and/or gating.[21,22] This work on $P2X_1R$ and $P2X_2R$ has been recently reviewed and the model for ATP binding domain has been suggested.[10] According to this model, the conserved positively charged residues K68, R292, and K309 ($P2X_1$ numbering) could be involved in coordination of negatively charged phosphate chain of ATP and the aromatic F185 and F291 could be associated with binding of adenine ring of ATP. The model also accommodates residues responsible for metal ion regulation of P2XRs, as well as pH sensitivity of receptors.

The extracellular loop of P2XRs contains a sequence stretch in positions 170 to 330 that exhibits similarities with the catalytic domains of a family of enzymes known as class II aminoacyl-tRNA synthetases, as shown by sequence alignments and secondary structure predictions.[23] From this comparison, five residues emerged

as potentially relevant for ATP binding at P2XRs: K190, K197, F230, D280, and R318 ($P2X_4$ numbering). Mutation studies with $P2X_2R$[19,20] are not incompatible with this model, whereas experiments with site-directed mutagenesis at $P2X_1R$ strongly argue against this model.[10] In our ongoing experiments, we are using the sequence and secondary structure similarities between the K180-K326 ectodomain region of $P2X_4$ and the class II aminoacyl-tRNA synthetases as a guide to generate a three-dimensional model of the receptor-binding site and to design mutants. The interplay between homology modeling and site-directed mutagenesis at the present stage of investigations suggests a model of ATP binding domain, where D280 residue coordinates ATP binding via the magnesium, F230 residue coordinates the binding of the adenine ring of ATP, and K190, H286, and R278 residues coordinate the actions of negatively charged α-, β-, and γ-phosphate groups, respectively.[24]

GATING AND IONIC CONDUCTION

The binding of ATP to ligand binding pocket leads to gating of P2XR, a key conformation change that enables the movement of cations through the pore of channels. It appears that the two transmembrane domains are in close proximity[25] and that both domains contribute to the formation of the pore.[3] The relevance of residues that line the channel walls on ion conductance was also studied.[26,27] All P2XR are cation-selective channels that show little selectivity for sodium over potassium. The

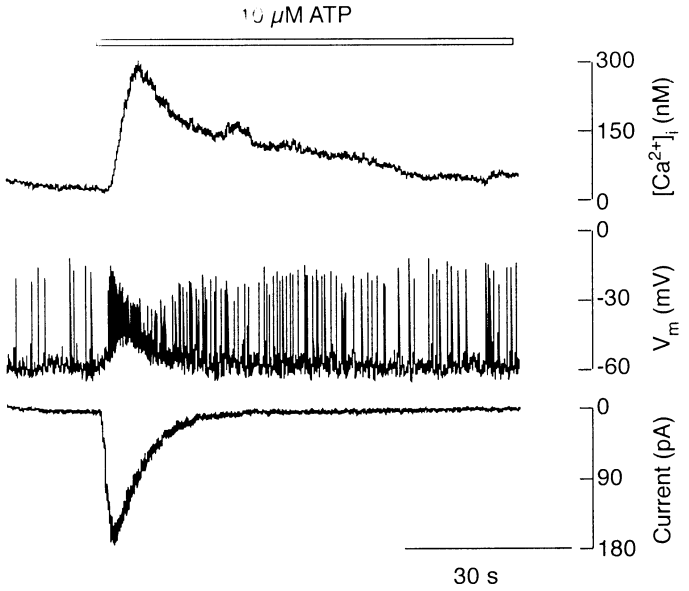


FIGURE 1. Modulation of electrical activity by activation of $P2X_{2b}R$ expressed in spontaneously firing GT1 neurons. The cell was initially stimulated with ATP for simultaneous membrane potential and calcium recordings, and then washed for 10 minutes and restimulated with the same dose of ATP for current recording when clamped at –90 mV. The horizontal bar above traces indicates the duration of ATP stimulation.

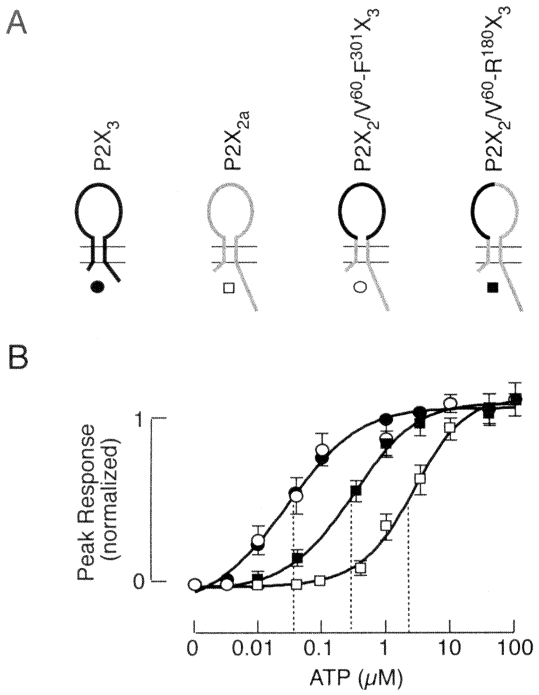
pores of P2XRs are permeable to Ca^{2+}, but receptors differ in their conductivity for this ion. $P2X_2R$, $P2X_4R$, and $P2X_7R$ are also permeable to larger organic cations, such as NMDG, Tris, and TEA, and permeability increases with time, a phenomenon known as pore dilation,[3] indicating the existence of several open conformation states of P2XR. Some progress was also made in characterizing the transduction of signaling from the ligand-binding pocket toward the other regions of molecule. Vial and colleagues[10] suggested the relevance of P225 and G247 in conformation changes associated with ATP binding, and our study[24] indicated the relevance of R318 of $P2X_4R$ in signal transduction toward the second transmembrane domain.

In non-excitable cells, Ca^{2+} influx through the pore represents the main mechanism by which P2XRs modulate cellular functions. In excitable cells, activated P2XRs also generate global Ca^{2+} signals by depolarizing cells and facilitating voltage-sensitive Ca^{2+} influx.[28,29] Thus, Ca^{2+} influx through the plasma membrane channels and its diffusion within the cells provide an effective mechanism by which P2XRs control numerous cellular functions in different cellular compartments. FIGURE 1 illustrates the temporal relationship between activation of transmembrane current, modulation of frequency of action potentials, and generation of global Ca^{2+} signals. The peak amplitude and the temporal aspects of current and Ca^{2+} signaling are receptor specific. Parallelism in current and calcium signaling patterns observed under different experimental conditions indicate the potential application of single-cell calcium measurements in studies of P2XR activity.[28] There are several advantages of single-cell calcium measurements in such studies, including the number of cells that can be examined simultaneously, the preserved interior of the cells compared to the whole-cell patch-clamp recording, and the possibility to study the spatial aspects of signaling and physiological role of P2XRs in intact cells. However, this method has its limitations, including the temporal dissociation between calcium signaling and P2XR activity and the selection of cell model with respect to the endogenous expression of P1Rs and P2YRs, the coupling of calcium influx to calcium-induced calcium release from intracellular stores, and the cell type–specificity in pathways controlling calcium efflux.[28]

DEACTIVATION OF P2XRS

As shown in Scheme **1**, there are two scenarios for deactivation of non-desensitizing P2XRs: (1) a channel-inherent slow transition from open to closed state (k_4) followed by a faster dissociation of agonist from receptor (k_2) or (2) fast channel closure, followed by slow dissociation of agonist from receptor. The experimentally observed time-course of current deactivation ultimately reflects agonist unbinding, but it does not clarify which transition, k_4 or k_2, is the rate-limiting step for this process.[12] Rettinger and Schmalzing first observed that potency of several agonists to activate a non-desensitizing $P2X_2/P2X_1$ chimera inversely correlates with the corresponding time constant of deactivation.[30] Based on this and other observations, they suggested that deactivation of P2XRs reflects predominantly the dissociation of agonist from receptor (k_2), although they lacked information about the deactivation properties of parental P2XRs.

To further dissect the mechanism of deactivation, we used $P2X_2R$ and $P2X_3R$ as parental receptors and generated two $P2X_2/P2X_3$ chimeras: $P2X_2$/V60-F301X_3,



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Receptor	EC_{50} (nM)	τ_{off} (s)
$P2X_3$	39 ± 7	0.9 ± 0.1
$P2X_2$/V60-F301X_3	48 ± 6	40.8 ± 3.8
$P2X_2$/V60-R180X_3	352 ± 18	0.9 ± 0.2
$P2X_2$	2618 ± 297	0.29 ± 0.4

FIGURE 2. Potency of agonists at parental and chimeric P2XRs. (**A**) Schematic representation of the wild type and chimeric constructs. (**B**) Concentration dependence of ATP-induced peak response in cells expressing wild type $P2X_3$ (●), wild type $P2X_2$ (□), chimeric $P2X_2$/V60-F301X_3 (○), and chimeric $P2X_2$/V60-R180X_3 (■) receptors. Data shown are means ± SEM and the peak amplitudes to 100 μM ATP application were used to normalize responses. (**C**) The lack of correlation between EC_{50} and τ_{off} values for parental and chimeric receptors in response to stimulation with ATP. The ATP concentrations used in the experiments to generate τ_{off} values were: 0.1 μM ATP for $P2X_3$R, 1 μM ATP for $P2X_2$/V60-F301X_3, 10 μM for $P2X_2$/V60-R180X_3, and 100 μM for $P2X_2$R.

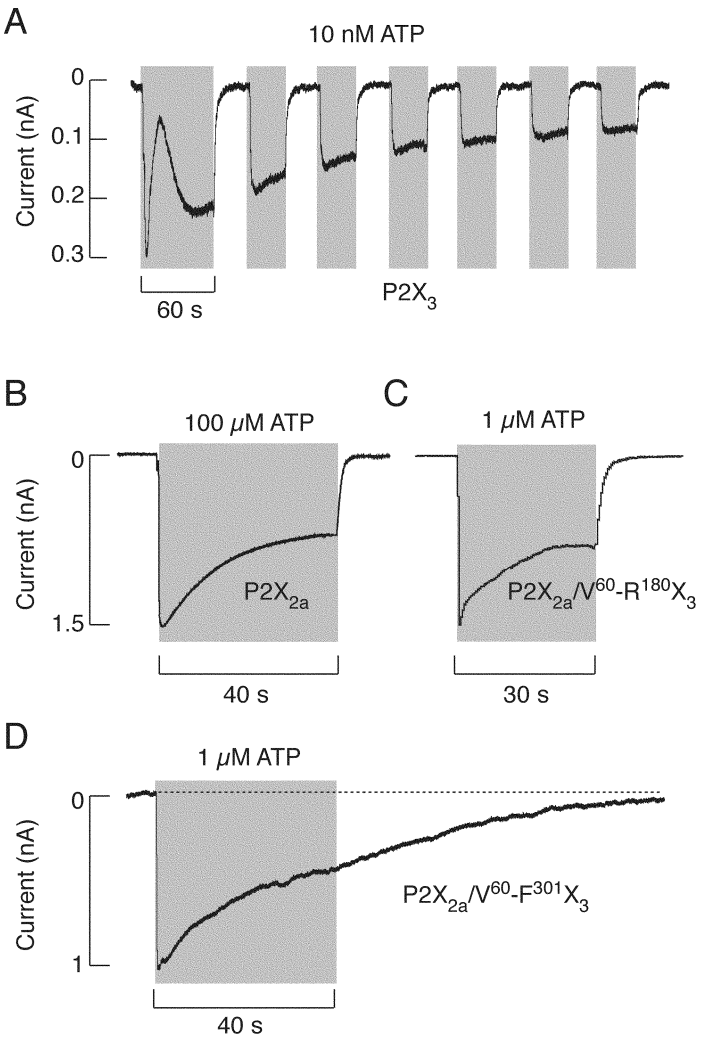

FIGURE 3. Deactivation properties of parental and chimeric receptors. (**A**) Pattern of current signaling during repetitive stimulation of $P2X_3R$-expressing cells with 10 nM ATP. (**B**) Typical traces of $P2X_2R$ current induced by 40 sec application of 100 μM ATP. (**C**) Current signaling by $P2X_2/V60\text{-}R180X_3$ chimera induced by 30 sec application of 1 μM ATP. (**D**) The lack of obvious deactivation of $P2X_2/V60\text{-}F301X_3$ chimera after removal of 1 μM ATP.

containing the extracellular domain from V60 to F301 of $P2X_3R$ instead of the native I66-Y310 sequence of $P2X_2R$, and $P2X_2$/V60-R180X_3 chimera containing the V60-R180 sequence of $P2X_3R$ instead of I66-H192 sequence of $P2X_2R$ (FIG. 2A). When expressed in HEK293 cells under identical experimental conditions, parental $P2X_2R$, and $P2X_3R$ responded to ATP with a rapid rise in current, with peak amplitude dependent on agonist concentrations. The estimated EC_{50} for $P2X_2R$ and $P2X_3R$ were 3 μM and 39 nM, respectively (FIG. 2, B and C). The potency of ATP at $P2X_2$/V60-F301X_3 chimera was comparable to that of parental $P2X_3R$. On the other hand, the EC_{50} for $P2X_2$/V60-R180X_3 chimera was between those at $P2X_3R$ and $P2X_2R$ (FIG. 2, B and C). These results, discussed in details in Zemkova and colleagues[31] indicate that the V60-F301 ectodomain sequence of $P2X_3R$ accounts for the ligand-selective profile of $P2X_3R$ and that both N- and C-halves of this sequence contribute to the efficacy of agonists.

In contrast to $P2X_1R$, it is possible to estimate the deactivation properties of $P2X_3R$ current, when receptors were stimulated with non-desensitizing ATP concentrations (FIG. 3A). Both the decline in current after removal of agonist and the rise in current after re-stimulation of cells indicate that the time constant of current decay (τ_{off}) reflects deactivation of this receptor. The estimated τ_{off} values for $P2X_3R$ were between 0.5 sec and 0.9 sec, depending on ATP concentration. $P2X_2R$ current also declines rapidly after removal of ATP and the estimated τ_{off}s after 60-sec stimulation with ATP were between 0.2 sec and 0.3 sec, in the range observed by others. The $P2X_2$/V60-R180X_3 chimera also deactivated following a 30-sec application of 1 μM ATP (FIG. 3C) with τ_{off}s between 0.7 and 1 sec. On the other hand, $P2X_2$/V60-F301X_3 chimera differed from parental receptors with respect to the decay of response after removal of agonist. Practically, $P2X_2$/V60-F301X_3 current declined to zero during 2-min after exposure to 1 μM ATP for 40 sec. Furthermore, no obvious change in the profile of current was observed after removal of ATP in these chimera-expressing cells (FIG. 3D).

Thus, although ATP activates $P2X_3R$ and $P2X_2$/V60-F301X_3 chimera equipotently, there was more than a 65-fold difference in the rates of current decay between parental and chimeric receptors after agonist removal, indicating that the chimerization-induced shift in EC_{50} values for ATP does not result in a proportional shift in rates of receptor deactivation. Furthermore, a decrease in the potency of ATP for $P2X_{2a}$/V60-R180X_3 chimera compared to $P2X_3R$ was not accompanied with a decrease in τ_{off} (FIG. 2C). The lack of correlation between EC_{50} and τ_{off} values for parental and chimeric receptors was also observed in response to other agonists.[31] These results are consistent with a hypothesis that agonist dissociation from its binding site does not represent the rate-limiting step for receptor deactivation. Our findings are more in accordance with the general view for ligand-gated receptor channels where conformation changes associated with gating do not allow agonist dissociation.[32] We further suggest that the stability of open conformation state for P2XRs reflects on deactivation kinetics and dissociation of agonist from receptors.[31]

STRUCTURAL DETERMINANTS OF RECEPTOR DESENSITIZATION

P2XRs differ in their desensitization rates: $P2X_1R$ and $P2X_3R$ desensitize rapidly, $P2X_4R$ and $P2X_5R$ desensitize with moderate rates, whereas $P2X_2R$, $P2X_6R$, and

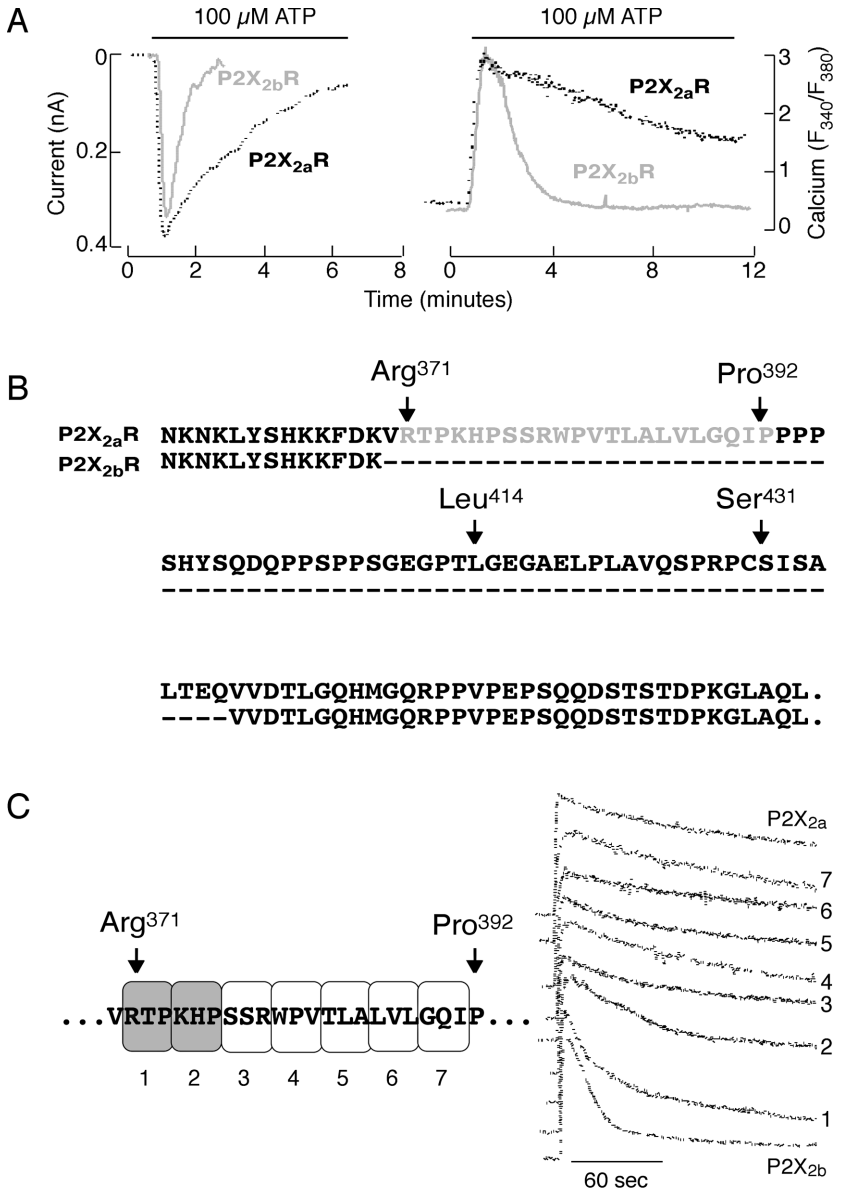

FIGURE 4. Identification of the C-terminal amino acid residues contributing to desensitization of $P2X_2R$. (**A**) Typical patterns of current (*left*) and calcium (*right*) signaling by full-size receptor ($P2X_{2a}R$) and spliced variant ($P2X_{2b}R$). (**B**) The amino acid sequences of the C termini of $P2X_{2a}R$ (*upper line*) and $P2X_{2b}R$ (*bottom line*). *Dashed lines* indicate the deleted segment in $P2X_{2b}R$. The residue of $P2X_2R$ indicated by the *arrows* are changed to in-frame stop codons. Residues in the *gray letter* comprise a critical region for sustained activity of receptor. (**C**) Calcium influx through triple alanine replacement mutants of $P2X_{2a}R$. (*Left*) Schematic representation of the mutated segments in C terminus of $P2X_2R$. The boxes illustrate the residues that were replaced with alanine in each mutant. (*Right*) Calcium influx responses of $P2X_{2a}R$, $P2X_{2b}R$, and seven $P2X_{2a}R$ C-terminal mutants evoked by 100 μM ATP.

$P2X_7R$ desensitize slowly or do not desensitize.[3] Two complementary hypotheses emerged from previous work on desensitization of P2XRs, one based on the actions of intracellular messengers and the other based on the structure of channels. The intracellular domains of P2X receptors contain several consensus sites for phosphorylation. The site-directed mutagenesis experiments indicated the relevance of a highly conserved N-terminal site as a substrate for protein kinase C in functional desensitization of receptors. This site appears to be phosphorylated constitutively.[33–35] Phosphorylation of a protein kinase A site in C-terminal of $P2X_{2a}R$ may also participate in control of receptor desensitization.[36] Thus, both G_q- and G_s-coupled seven transmembrane domain receptors could influence the conductivity of P2XRs.

The discovery and characterization of spliced form of $P2X_2R$, termed $P2X_{2b}R$, revealed a potential importance of C-terminal domain residues in control of receptor desensitization.[37–39] As shown in FIGURE 4A, the lack of V370-Q438 C-terminal amino acid sequence in $P2X_{2b}R$ is associated with the rapid desensitization of these channels.[39] To identify the structural elements in the full-size receptor ($P2X_{2a}R$) C-terminus that are responsible for prolonged opening of channels, initially we generated a series of C-terminal truncated mutants (FIG. 4B). These studies revealed the potential importance of the R371-P392 region in control of receptor desensitization. Subsequently, triple alanine replacement and single amino acid deletion mutants were constructed to identify precisely the amino acid sequence that is critical for long-lasting signaling by $P2X_{2a}R$ (FIG. 4C, left panel, gray boxes). Finally, spliced amino acids from the C-terminus of the wild type channel were gradually added to $P2X_{2b}R$ to regain the slow desensitizing pattern of Ca^{2+} signaling in response to prolonged agonist stimulation.[40] The results of this investigation indicate that a polypeptide region containing R371-P376 sequence residues is the sequence important for delay in desensitization of $P2X_{2a}R$.

This 6-amino-acid–region is located near the second putative transmembrane domain and the C-terminal difference in amino acid sequences among the members of P2XRs starts from this region. To study the possible role of the structural diversity of this region in the control of receptor desensitization, the R371-P376 sequence of $P2X_{2a}R$ was introduced to $P2X_3R$ and $P2X_4R$ instead of the native T362-K367 and E376-G381 sequences, respectively. The reverse mutations were also performed at $P2X_{2a}R$ C-terminus by substituting the R371-P376 sequence with the corresponding 6-amino-acid sequence of $P2X_1R$, $P2X_3R$, and $P2X_4R$. In cells expressing $P2X_3R$ or $P2X_4R$ mutant, the desensitization rate of ATP-induced Ca^{2+} response was slower than that of wild type receptors. On the other hand, all chimeric $P2X_{2a}R$ subunits formed functional channels in GT1 cells that exhibited enhanced desensitization rates when compared to the wild type $P2X_{2a}R$.[8] These results indicate that the R371-P376 sequence of $P2X_{2a}R$ and the equivalent sequences of $P2X_3R$ and $P2X_4R$ are important in determining the desensitization to ATP stimulation.

Our experiments have also suggested that the ectodomain of P2XRs not only contains agonist binding site, but also controls desensitization via its coupling to the C-terminal domain.[7,14,41] The main conclusion that emerged from this work is that P2XR desensitization pattern is a receptor- and ligand-specific. The receptor-specific desensitization patterns were observed in response to ATP, the native agonist for these channels, but were less obvious when stimulated with 3′-O-(4-benzoyl)benzoyl-ATP (BzATP), and were lost when receptors were stimulated with αβ-meATP. Increase in EC_{50}s for all three agonists induced by substituting the ectodomains in-

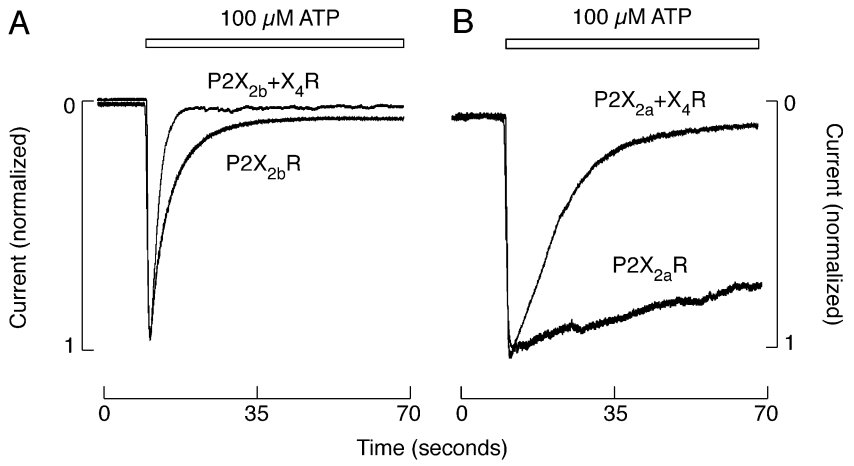

FIGURE 5. Acceleration of $P2X_{2a}R$ and $P2X_{2b}R$ desensitization by substituting their common ectodomain with the $P2X_4R$ ectodomain. The traces shown are representative for $P2X_{2b}R$ and chimeric $P2X_{2b}$+X_4R (**A**) and $P2X_{2a}R$ and chimeric $P2X_{2a}$+X_4R (**B**).

dicates that the potency of agonists reflects the ligand specificity of receptor desensitization, i.e., highly potent agonists trigger the subtype-specific desensitization pattern, whereas desensitization induced by agonists with lower potency is less receptor specific. Our work also suggested that the C-terminal-dependent desensitization pattern was preserved in chimeric $P2X_{2a}/X_4$ and $P2X_{2b}/X_4$ channels (FIG. 5). In accordance with our data on $P2X_4R$, others have shown that a single mutation in the extracellular domain leads to a decrease in agonist potency of $P2X_3R$, as well as to a decrease in the rate of receptor desensitization.[42] Thus, there is a complex interplay between the agonist binding sites and the extra and intracellular domains of P2XRs controlling the onset of desensitization.

RECOVERY FROM DESENSITIZATION OF P2XRS

In contrast to numerous studies addressing the dependence of desensitization rates on receptor structure, the relationship between the molecular organization of receptors and recovery from desensitization has not been studied extensively. The protocol we used to estimate the recovery time is shown in FIGURE 6 and discussed in detail elsewhere.[31] Cells expressing $P2X_{2b}R$ and $P2X_3R$ were stimulated repetitively with ATP pulses, with a progressive increase in the interpulse intervals. The initial 60-sec ATP pulse was sufficient to almost completely desensitize both receptors. The recovery of $P2X_{2b}R$ from desensitization occurred mono-exponentially, with a time constant (τ_{rec}) of 55 sec, whereas the $P2X_3R$ recovered with τ_{rec} of 141 sec. Others have also observed recovery of $P2X_3R$ with similar τ_{rec}.[43] The same authors also observed that receptors exhibiting comparable sensitivity to agonists with

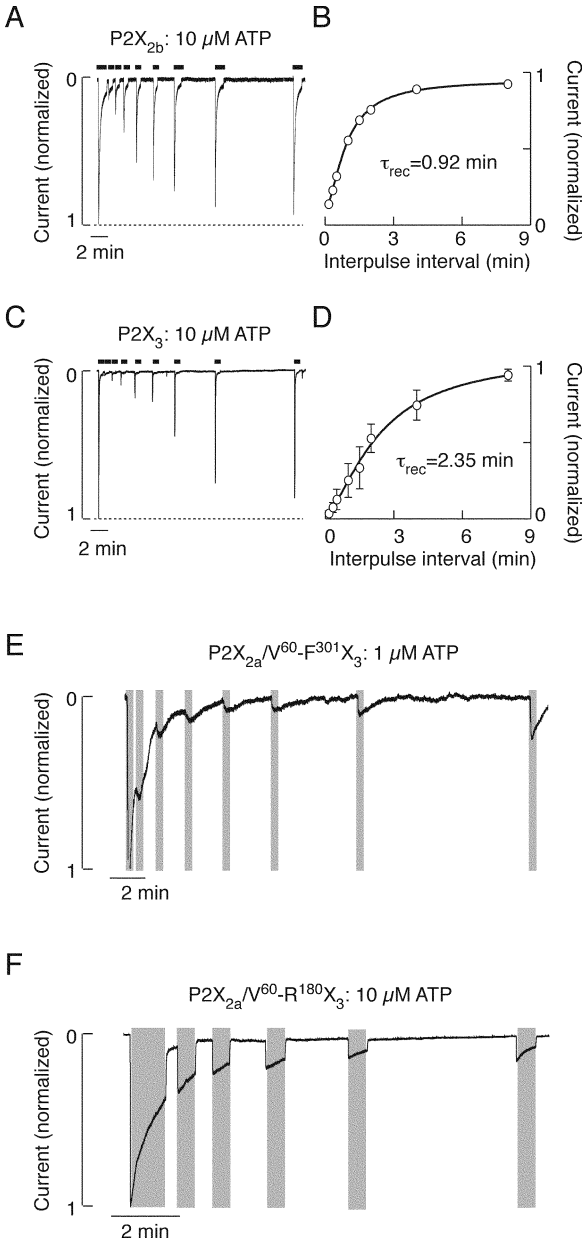

FIGURE 6. Recovery from desensitization of parental and chimeric receptors. (**A** and **C**) The example records showing $P2X_{2b}$ (**A**) and $P2X_3$ (**C**) current desensitization during 60 sec application of 10 μM ATP and recovery of response to ATP after different washout times. The *bars* above traces indicate time of ATP application. (**B** and **D**) Recovery of responses to ATP in $P2X_{2b}R$ (**B**) and $P2X_3R$ (**D**) expressing cells, shown as the ratio peak/first peak response and plotted as a function of washing time. (**E**) Patterns of $P2X_2$/V60-F301X_3 current signaling during repetitive stimulation with 1 μM ATP. (**F**) $P2X_2$/V60-R180X_3 current signaling induced by repetitive application of 10 μM ATP. *Gray areas* indicate the duration of agonist application.

respect to activation and onset of desensitization show different rates of recovery from desensitization. This observation raised the possibility that recovery of P2XRs is governed by independent mechanisms from that controlling receptor activation and desensitization. In addition, our observation that parental receptors recovered from desensitization completely, and in a relatively short period, suggests that internalization, which is a receptor-specific phenomenon and usually requires longer time periods,[44,45] probably does not significantly participate in $P2X_2R$ and $P2X_3R$ desensitization and recovery from desensitization.

Experiments with chimeric receptors provided some insights into the structural determinants of recovery from desensitization.[31] As shown in FIGURE 6E, repetitive stimulation of $P2X_2$/V60-F301X_3 chimera with 30-sec pulses of 1 μM ATP led to a progressive decrease in response. The current did not decline to basal level during the first three pulses, indicating continuous desensitization of receptors between ATP stimuli. Receptors showed only a partial recovery of response with increase in the interpulse periods, and the full recovery of response was never reached within up to 40 minutes of washing period. Interestingly, the recovery from desensitization state of $P2X_2$/V60-R180X_3 chimera, which deactivated more rapidly than $P2X_2$/V60-F301X_3 chimera, was also dramatically extended. FIGURE 6F illustrates that recovery of a non-desensitized component of $P2X_2$/V60-R180X_3 current was fast, whereas the interpulse intervals between 30 sec and 4 min were practically ineffective in recovery of desensitizing component. As with $P2X_2$/V60-F301X_3 chimera, the extension of washing periods up to 40 minutes was not sufficient for full recovery from desensitized state. This finding suggests the relevance of N-half of ectodomain in conformation changes associated with desensitization. Since $P2X_3R$ has the same sequence, but fully recovers within 10–15 minutes, these results further indicate that the intramolecular interactions between N-half of ectodomain sequence and other regions of the same subunits or between the subunits account for the specificity of receptor action.[31] The site-directed mutagenesis confirmed the relevance of several ectodomain residues in recovery from desensitization of $P2X_3R$.[42]

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